



Divergence in calls but not songs in the orchard oriole complex: *Icterus spurius* and *I. fuertesi*

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Birdsong has important functions in attracting and competing for mates, and song characteristics are thought to diverge rapidly during the process of speciation. In contrast, other avian vocalizations that may have non-reproductive functions, such as calls, are thought to be more evolutionarily conserved and may diverge more slowly among taxa. This study examines differences in both male song and an acoustically simpler vocalization, the 'jeet' call, between two closely related taxa, *Icterus spurius* and *I. fuertesi*. A previous study comparing song syllable type sharing within and between *I. spurius* and *I. fuertesi* indicated that their songs do not differ discernibly. Here we measured 18 acoustic characteristics of their songs and found strong evidence supporting this prior finding. In contrast, we measured 17 acoustic characteristics of jeet calls and found evidence of significant divergence between the two taxa in many of these characteristics. Calls in *I. fuertesi* have a longer duration, a larger frequency bandwidth, a lower minimum frequency, a lower beginning frequency, and greater levels of both frequency and amplitude modulation in comparison to the calls of *I. spurius*. In addition, *I. fuertesi* calls contain two distinct parts, while the calls of *I. spurius* have only one part. Thus, we find evidence of divergence in the calls of the two taxa but not their songs challenging the widespread assumption that complex bird song evolves more rapidly than other types of vocalizations. Understanding divergence in multiple vocalization types as well as other behavioral, morphological, and molecular traits is important to understanding the earliest stages of speciation.

Premating isolation can result from a rapid divergence in traits influenced by sexual selection (Grant and Grant 1997, T. Price 1998). Birdsong, which can play an important role in both territory defense and in mate choice, can evolve through both sexual selection and ecological adaptation (Grant and Grant 1997, T. Price 1998, Shaw and Parsons 2002, Kroodsma 2004, Ritchie 2007, Collins et al. 2009). Song is thought to diverge rapidly and relatively early in the process of speciation, before other barriers to mating, such as hybrid incompatibility, arise (Slabbekoorn and Smith 2002, Mendelson 2003, Edwards et al. 2005, Balakrishnan and Sorenson 2006, Mendelson et al. 2007). In songbirds, songs are generally learned and can change rapidly through cultural transmission, for example through copy errors (Grant and Grant 1997, T. Price 1998, Podos et al. 2004). Recent studies of closely related taxa have used analyses of song divergence, in conjunction with molecular analyses, to help delimit species boundaries (Irwin 2000, Balakrishnan and Sorenson 2006, Seddon and Tobias 2007, Brambilla et al. 2008, Seddon et al. 2008, Dingle et al. 2010). Song comparisons are especially useful in studies where neutral molecular markers of the taxa under consideration have not yet had time to achieve reciprocal monophyly (Joseph and Omland 2009). Moreover, studies of song within and between closely related taxa can be helpful in understanding the role vocal

divergence may play in the context of establishing species boundaries.

The orchard oriole complex is made up of two taxa: the orchard oriole *Icterus spurius*, which breeds across eastern North America from Canada to central Mexico, and Fuertes' oriole *I. fuertesi*, which breeds in a narrow strip of coastal lowlands of southern Tamaulipas and Veracruz, Mexico (Chapman 1911, Scharf and Kren 1996, Jaramillo and Burke 1999). *Icterus fuertesi* was originally described as a separate species of oriole in the early 1900s based on its unique adult male plumage as well as on purported differences in size and in song from that of its closest relative, *I. spurius* (Chapman 1911). The status of *I. fuertesi* was later changed to that of a subspecies within the orchard oriole complex based largely on a study that showed the size differences were not significant (Graber and Gruber 1954). These taxa also share many morphological characteristics, such as female and juvenile plumage coloration (Gruber and Gruber 1954, Hofmann et al. 2007, Kiere et al. 2007).

More recent studies have suggested the two taxa may be less closely related. While they were lumped partly due to alleged high levels of within-taxon variation in adult male plumage coloration, rigorous spectrophotometric studies of plumage coloration do not show any overlap in color variation and show fixed color differences between them (Hof-

mann et al. 2007, Kiere et al. 2007). *Icterus spurius* is a long distance migrant, overwintering from southern Mexico to northern South America, while *I. fuertesi* is a short distance migrant, overwintering in the southern portion of its breeding range (where it likely overlaps with *I. spurius*) (Jaramillo and Burke 1999, Tobóm-Sampedro and Rojas-Soto 2014). The two taxa breed at different latitudes and have different bioclimatic niches for their breeding distributions (Martin and Omland 2011). Comparisons of their genetics support a very recent divergence between the taxa – so recent, in fact, that even their mitochondrial DNA (mtDNA) lacks reciprocal monophyly (Baker et al. 2003, Sturge 2013). However, both their mtDNA and their nuclear DNA (locus TGFB2 – Sturge 2013) show evidence of population structure between the taxa, supporting their characterization as two evolutionarily distinct groups. This distinctiveness is further supported by the lack of described hybrids in nature and the fact that the breeding ranges of the two taxa are allopatric with respect to one another (Chapman 1911, Gruber and Gruber 1954, Scharf and Kren 1996, Jaramillo and Burke 1999, Tobóm-Sampedro and Rojas-Soto 2014).

A study of song variation across orioles (Price et al. 2007), including the orchard oriole (but not Fuertes' oriole), found that the orchard oriole was very different from its closest relatives included in the study, with a relatively large number of vocal changes occurring since the node joining orchard orioles to a Caribbean and South American clade. This result suggests that the song of orchard orioles has changed considerably since its lineage split from the rest of the orioles in the *Icterus* phylogeny. Song is thus a potentially useful character for testing divergence between the orchard oriole and its sister taxon, Fuertes' oriole. While an early account claimed that the vocalizations of the two taxa were discernibly different to the human ear (Chapman 1911), a more recent study examining levels of song syllable type sharing within and between the two taxa found no evidence of differentiation in syllable use between their songs (Hagemeier et al. 2012).

Like other oscine passerines, orioles produce a range of vocalizations other than songs, usually termed calls (Jaramillo and Burke 1999). *Icterus fuertesi* and *I. spurius* males responding to territorial intrusions often produce a loud, frequency-modulated vocalization hereafter referred to as a 'jeet' call. Call repertoires of birds generally can serve a variety of functions such as contact, aggression, or predator alarm (Marler 2004a). Calls can show individual and geographic variation (Baker 2000, Bradbury et al. 2001, Marler 2004b, Sewall 2009, Benedict and Krakauer 2013). Some acoustic aspects of a call may also be plastic, allowing for call adjustment and learning through tutors (J. Price 1998, Marler 2004b, Sewall 2009). Because calls can become modified through both heritable genetic changes or through changes in cultural transmission (Marler 2004a), calls also have the potential to diverge as two taxa speciate. This divergence can be driven through natural and/or sexual selection, stochastic processes resulting from the original divergence event, and/or adaptation to local environmental conditions. Divergence in call structure can also be influenced by physiological constraints (Marler 2004a, Irwin et al. 2008, Benedict and Krakauer 2013, Wheatcroft and Price 2013). As a whole, calls have been largely understudied in avian

species compared with song – thus, there is a need for studies that examine both the role that calls play in terms of social context, as well as how calls diverge between closely related taxa, to gain a better understanding of how calls evolve over time (Marler 2004b, Irwin et al. 2008, Benedict and Krakauer 2013).

Our goal for this study was to measure temporal, frequency and amplitude characteristics of both song and jeet calls from across each taxon's range to look for evidence of vocal divergence between these two taxa. Jeet calls are often produced between bouts of singing for territory defense, and may serve a similar function as song. Thus, as both song and jeet calls are potentially shaped by sexual selection, they could diverge more rapidly than neutral genetic markers (Baker and Baker 1990, Baker 1994, Grant and Grant 1997, T. Price 1998, Mendelson 2003, Patten et al. 2004, Grant and Grant 2008, Seddon et al. 2008). Therefore, vocalizations such as songs and jeet calls can potentially help us better understand the divergence between *I. spurius* and *I. fuertesi*. This taxonomic complex, with its very recent divergence, offers us an intriguing case study in which we can examine divergence in vocal characters at the earliest stages of divergence.

Methods

Study sites

We obtained many of the *I. spurius* and one of the *I. fuertesi* recordings included in this study from the Macaulay Library of Natural Sounds (Cornell Univ., Ithaca, NY; Table 1 and 2). We further increased our sample size by conducting field work in Maryland and Texas, USA, and in Veracruz, Mexico. For *I. spurius*, field sites included parks and protected wildlife areas in Maryland and in Texas. For *I. fuertesi*, field sites spanned four locations across Veracruz, from Tamiahua in the north to Tlacotalpan in the south, and included hedgerows and tree-lined streets in and around human habitation, as well as the tree and shrub-lined edges of farmer's fields. We completed two full field seasons (2008 and 2009), collecting recordings of *I. spurius* in Maryland from late April to late May, and from mid-June until early July, and from *I. fuertesi* in late May–early June. Field recordings were made mainly from 06:00–13:00 DST and occasionally from 17:00–dusk of males vocalizing at territory posts. These recordings included both songs and jeet calls as territorial males produce both types of vocalizations while defending their territories (field observations; also supported by presence of jeet calls on many of the song recordings we obtained from the Macaulay Library of Natural Sounds (Cornell Univ., Ithaca, NY)). To increase our sample of jeet calls, we spent four days recording birds in Texas from 30 May to 2 June, 2013. Locations of all recordings used in this study can be seen in Fig. 1 and Table 1 (songs) and Table 2 (jeet calls). During our field seasons, we located vocalizing males and recorded each for a period of three to five minutes using a Marantz PMD 660 digital recorder (Marantz, Sagamihara, Japan) and a Sennheiser ME-62 microphone (Sennheiser Electronic, Wennebostel, Germany) mounted in a 32 cm radius parabolic reflecting dish (Telinga Microphones, Tobo, Sweden). Songs and calls were sampled and stored digitally as 'wav' files at 44.1 kHz.

Table 1. Recording location for adult male oriole songs used in comparison of vocalizations.

Species	Recordist	Location	Date
<i>Icterus spurius</i>	Robert Stein	Ogallala, Nebraska, USA	20 June 1961
<i>Icterus spurius</i>	Mark Robbins	Maryville, Missouri, USA	14 May 1991
<i>Icterus spurius</i>	Arthur Allen	Principia College, Illinois, USA	22 May 1954
<i>Icterus spurius</i>	Peter Kellogg	Spencer Nob, Ohio, USA	18 May 1954
<i>Icterus spurius</i>	Geoffrey Keller	Minnehaha Wildlife Mgt Area, Indiana, USA	25 May 1995
<i>Icterus spurius</i>	William Gunn	Point Pelee, Ontario, Canada	11 May 1954
<i>Icterus spurius</i>	William Gunn	Point Pelee, Ontario, Canada	22 May 1954
<i>Icterus spurius</i>	William Gunn	Point Pelee, Ontario, Canada	25 May 1956
<i>Icterus spurius</i>	George Reynard	Marleton, New Jersey, USA	13 May 1959
<i>Icterus spurius</i>	William Hershberger	Frederick County, Maryland, USA	25 April 1999
<i>Icterus spurius</i>	Rachel Sturge	Centennial Park, Maryland, USA	24 June 2009
<i>Icterus spurius</i>	Rachel Sturge	Schooley Mill Park, Maryland, USA	1 July 2009
<i>Icterus spurius</i>	Rachel Sturge	Cromwell Park, Maryland, USA	18 June 2008
<i>Icterus spurius</i>	George Reynard	Thomas, Georgia	12 May 1964
<i>Icterus spurius</i>	Geoffrey Keller	Bear Island Wildlife Mgt Area, S. Carolina, USA	2 June 1994
<i>Icterus spurius</i>	Oliver Hewitt	Huntington Beach State Park, S. Carolina, USA	10 June 1981
<i>Icterus spurius</i>	Geoffrey Keller	Big Bend National Park, Texas, USA	4 May 1986
<i>Icterus spurius</i>	Geoffrey Keller	Apalachicola Ntl Forest, Florida, USA	26 May 1988
<i>Icterus spurius</i>	Theodore Parker, III	Baton Rouge, Louisiana, USA	30 April 1992
<i>Icterus fuertesi</i>	L. Irby Davis	Mante, Tamaulipas, Mexico	20 May 1954
<i>Icterus fuertesi</i>	Rachel Sturge	Tamiahua, Veracruz, Mexico	6 June 2009
<i>Icterus fuertesi</i>	Rachel Sturge	Tamiahua, Veracruz, Mexico	6 June 2009
<i>Icterus fuertesi</i>	Rachel Sturge	Tamiahua, Veracruz, Mexico	6 June 2009
<i>Icterus fuertesi</i>	Rachel Sturge	Tuxpan, Veracruz, Mexico	4 June 2009
<i>Icterus fuertesi</i>	Rachel Sturge	Las Barrancas, Veracruz, Mexico	7 June 2008
<i>Icterus fuertesi</i>	Rachel Sturge	Las Barrancas, Veracruz, Mexico	7 June 2008
<i>Icterus fuertesi</i>	Rachel Sturge	Las Barrancas, Veracruz, Mexico	8 June 2008
<i>Icterus fuertesi</i>	Rachel Sturge	Las Barrancas, Veracruz, Mexico	8 June 2008
<i>Icterus fuertesi</i>	Rachel Sturge	Las Barrancas, Veracruz, Mexico	8 June 2008
<i>Icterus fuertesi</i>	Rachel Sturge	Las Barrancas, Veracruz, Mexico	8 June 2008
<i>Icterus fuertesi</i>	Rachel Sturge	Tlacotalpan, Veracruz, Mexico	30 May 2009
<i>Icterus fuertesi</i>	Rachel Sturge	Tlacotalpan, Veracruz, Mexico	31 May 2009
<i>Icterus fuertesi</i>	Rachel Sturge	Tlacotalpan, Veracruz, Mexico	1 June 2009
<i>Icterus fuertesi</i>	Rachel Sturge	Tlacotalpan, Veracruz, Mexico	1 June 2009
<i>Icterus fuertesi</i>	Rachel Sturge	Tlacotalpan, Veracruz, Mexico	2 June 2009
<i>Icterus fuertesi</i>	Rachel Sturge	Tlacotalpan, Veracruz, Mexico	2 June 2009

Table 2. Recording locations for jeet calls from male orioles.

Species	Recordist	Location	Date
<i>Icterus spurius</i>	Paul Driver	Palmyra, New Jersey, USA	6 May 2008
<i>Icterus spurius</i>	Curtis Marantz	Long Point State Park, New York, USA	17 May 1998
<i>Icterus spurius</i>	Rachel Sturge	Merkle Wildlife Sanctuary, Maryland, USA	22 May 2009
<i>Icterus spurius</i>	Rachel Sturge	Upper Marlboro, Maryland, USA	22 May 2009
<i>Icterus spurius</i>	Rachel Sturge	Schooley Mill Park, Maryland, USA	1 July 2009
<i>Icterus spurius</i>	Rachel Sturge	Halethorpe Farm Pond, Maryland, USA	29 June 2008
<i>Icterus spurius</i>	Rachel Sturge	Cromwell Park, Maryland, USA	23 June 2009
<i>Icterus spurius</i>	Rachel Sturge	Blackwater Wildlife Sanctuary, MD, USA	25 June 2009
<i>Icterus spurius</i>	Rachel Sturge	Ellis Bay, Maryland, USA	1 July 2008
<i>Icterus spurius</i>	Rachel Sturge	Ellis Bay, Maryland, USA	1 July 2008
<i>Icterus spurius</i>	Geoffrey Kelley	Minnehaha Wildlife Mgt Area, Indiana, USA	25 May 1995
<i>Icterus spurius</i>	Mike Nelson	Cove Lake State Park, Tennessee, USA	18 June 2008
<i>Icterus spurius</i>	Rachel Sturge	McFaddin Wildlife Sanctuary, Texas, USA	1 June 2013
<i>Icterus spurius</i>	Rachel Sturge	McFaddin Wildlife Sanctuary, Texas, USA	1 June 2013
<i>Icterus spurius</i>	Rachel Sturge	Sabine Woods, Texas, USA	31 May 2013
<i>Icterus spurius</i>	Rachel Sturge	Sabine Woods, Texas, USA	1 June 2013
<i>Icterus fuertesi</i>	Rachel Sturge	Tamiahua, Veracruz, Mexico	5 June 2009
<i>Icterus fuertesi</i>	Rachel Sturge	Tamiahua, Veracruz, Mexico	5 June 2009
<i>Icterus fuertesi</i>	Rachel Sturge	Tamiahua, Veracruz, Mexico	6 June 2009
<i>Icterus fuertesi</i>	Rachel Sturge	Las Barrancas, Veracruz, Mexico	7 June 2008
<i>Icterus fuertesi</i>	Rachel Sturge	Las Barrancas, Veracruz, Mexico	8 June 2008
<i>Icterus fuertesi</i>	Rachel Sturge	Tlacotalpan, Veracruz, Mexico	8 June 2008
<i>Icterus fuertesi</i>	Rachel Sturge	Tlacotalpan, Veracruz, Mexico	31 May 2009
<i>Icterus fuertesi</i>	Rachel Sturge	Tlacotalpan, Veracruz, Mexico	1 June 2009
<i>Icterus fuertesi</i>	Rachel Sturge	Tlacotalpan, Veracruz, Mexico	2 June 2009

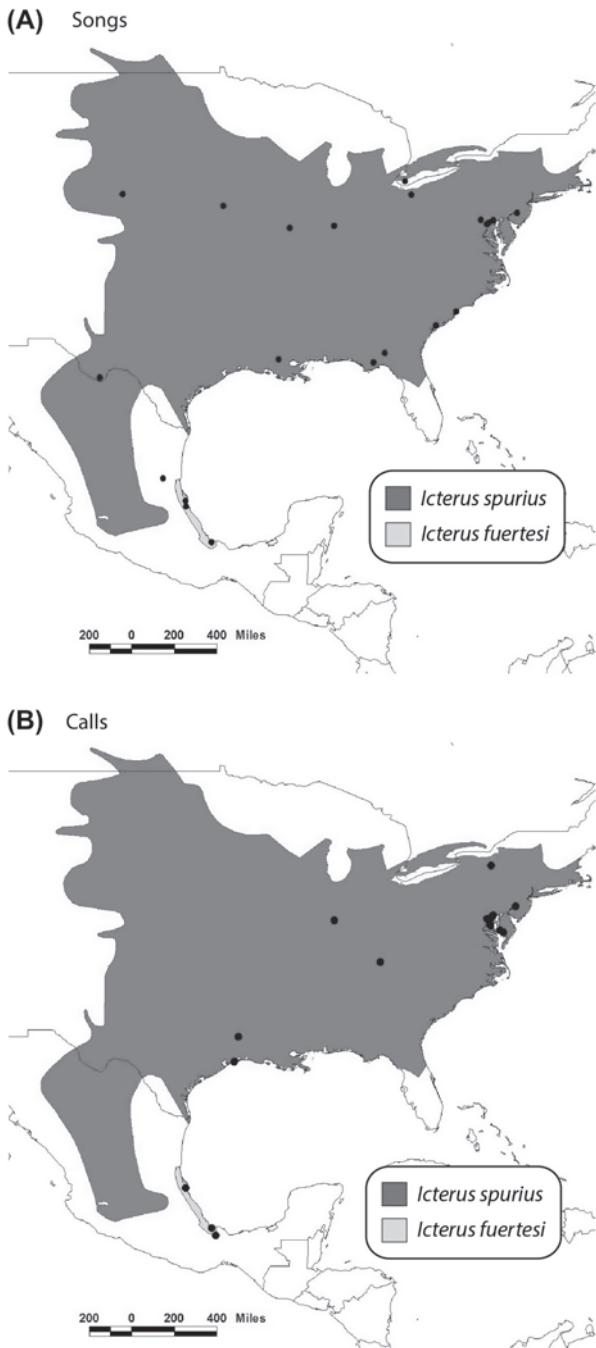


Figure 1. Recording locations for *I. spurius* and *I. fuertesi* songs (A) and jeet calls (B) measured in this study. This map also shows the breeding ranges for both species.

Song analysis

We compared the songs from ten *I. spurius* and nineteen *I. fuertesi*. Recordings were imported into RAVEN 1.1 (Cornell Univ., Ithaca, New York) sound analysis software to generate spectrograms using a 256 pt FFT; frequency resolution 188 Hz (for representative songs, see Fig. 2). To allow comparison with prior work on *I. spurius*, we measured 18 of the acoustic song characteristics used by Price et al. (2007) that were present in the songs of our focal taxa from spectrograms, using one sample song from each male oriole

included in our study (Table 3). Price et al. (2007), Table 1, provides more details on how these characteristics were measured. Note that unlike Price et al. (2007), which assigned their song characters scores or ranks in order to create categories to compare many taxa at once, we used the original values (e.g. frequency (Hz) or time (s)) for our comparison of only two taxa. As males were not color-banded, we excluded songs recorded within 200 m of each other unless we were certain that we were recording two different males (by locating the original male while recording a second male in an area). We also excluded songs that were recorded within 500 m on subsequent days or within 1 km in subsequent years, to reduce our chances of accidentally recording the same male twice.

Call analysis

We compared the jeet calls from sixteen *I. spurius* and nine *I. fuertesi*, and used SIGNAL sound analysis software (Beaman 2009) to measure variation in these calls to facilitate the finer-scale measurements of frequency and amplitude modulation in this acoustically simpler vocalization (for representative calls, see Fig. 3). Initial visual analyses of song spectrograms suggested no obvious acoustic differences between the taxa. In contrast, visual inspection of call spectrograms indicated several likely differences between the calls of the two taxa, thus focusing our subsequent analyses on this simple call note. For this analysis we searched through song recordings to locate and sample interspersed jeet calls. We also conducted an additional field season in Texas in 2013 (see above) to increase our sampling from the southern half of *I. spurius*'s range (Fig. 1B, Table 3). In general, jeet calls consisted of a single pure tone that decreased and/or increased in frequency over the course of 150–300 ms. In some cases the call was relatively unmodulated and of approximately constant amplitude throughout its duration; in other cases the call contained either frequency and/or amplitude modulation with or without a pronounced drop or rise in amplitude during the middle to last third of the call (see *I. fuertesi* calls, Fig. 3).

To characterize acoustic variation in the jeet call we initially measured overall call duration, and mean call frequency using a 16K pt FFT; 200 Hz smoothing width (frequency resolution 2.93 Hz). We then measured the mean frequency at the beginning, middle and end of each call (20 ms intervals at each call position, 1K pt FFT; frequency resolution 46.88 Hz; 200 ms smoothing width), and calculated the frequency ratios of the beginning/middle, middle/end, and beginning/end frequencies for each call. We used the beginning and end frequencies to calculate an overall frequency slope for the call. Calls were relatively narrow-band and limited to a small range of frequencies, and typically consisted of one element and no pauses (Fig. 3, Table 4). To examine relative amplitudes within calls we measured RMS (root-mean-square) amplitude at the beginning, middle, and end of jeet calls, and calculated amplitude ratios for the beginning/middle, middle/end, and beginning/end of each call. The acoustic characteristics we measured for jeet calls differed in part from those in our song analysis as a number of the song measurements we used (number of notes, number of pauses, other measures of a similar nature) involved

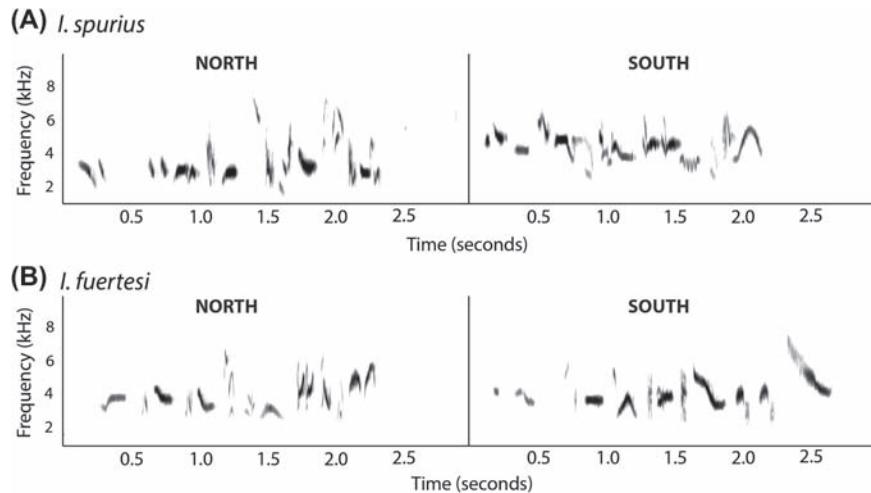


Figure 2. (A) Examples of *I. spurius* songs from the northern (Maryland, USA) and the southern (Texas, USA) populations included in this study. (B) Examples of *I. fuertesi* songs from the northern (north Veracruz, Mexico) and the southern (south Veracruz, Mexico) populations included in this study.

multiple acoustic elements, and could not be applied to the calls. However, there was considerable overlap in some of the more general measurements of duration and frequency in individual elements of song and the jeet call, including: duration of longest and average note, overall peak frequency (frequency of highest amplitude), maximum and minimum frequency (highest or lowest frequency measured throughout note or call), frequency range (max minus min frequency), and frequency slope (see values indicated in bold in Table 3 and 4).

As a way of generating an index of frequency modulation within each jeet call, which appeared to differ by eye across taxa in spectrograms (Fig. 3), we used the SIGNAL software to extract a spectral contour from a spectrogram of the call (256 pt FFT; 187.5 Hz frequency resolution, 5.3 ms time resolution). We then subtracted the overall frequency slope of

the call, removed any remaining DC offset, and then calculated the standard deviation of frequency across the remaining spectral contour (Beaman 2009). We also measured the maximum and minimum frequency of the call using this spectral contour, and calculated a frequency range across the call (in Hz) by subtracting the minimum frequency from the maximum frequency. We evaluated amplitude modulation by rectifying the amplitude envelope of the signal and tracking the envelope with a 5 ms exponential decay. We then calculated the standard deviation of variation in the amplitude envelope over the duration of the call. Finally, we visually identified from the spectrogram whether jeet calls had a pronounced amplitude drop or rise within the call, as such calls would appear two-part, rather than as a single tracing, on spectrograms (using a dynamic range of -6 dB to -40 dB from peak).

Table 3. Untransformed means of song characteristics measured for male *I. spurius* and *I. fuertesi*. Standard deviations are given in parentheses. Characteristics in bold are analogous to similar measures for the jeet call (Table 4).

Song characteristic	<i>I. spurius</i>	<i>I. fuertesi</i>		
Song duration (s)	2.7	(\pm 0.6)	2.5	(\pm 0.3)
Percentage of notes in song (%)	72.8	(\pm 8.9)	67.8	(\pm 7.0)
Percentage of note overlap (%)	4.2	(\pm 3.6)	3.9	(\pm 4.5)
Duration of longest note (ms)	237.3	(\pm 59.0)	232.4	(\pm 32.4)
Duration of longest pause (ms)	115.4	(\pm 65.3)	138.4	(\pm 54.8)
Average note duration (ms)	69.8	(\pm 17.1)	67.4	(\pm 13.6)
Average pause duration (ms)	50.4	(\pm 13.8)	54.0	(\pm 11.0)
Pause rate (pause/s)	6.4	(\pm 1.3)	6.8	(\pm 1.2)
Overall peak frequency (Hz)	4142.6	(\pm 819.7)	4001.2	(\pm 870.0)
Maximum frequency across song (Hz)	6991.4	(\pm 999.0)	7287.3	(\pm 1180.8)
Minimum frequency across song (Hz)	1158.9	(\pm 283.2)	1226.3	(\pm 219.4)
Frequency range across song (Hz)	5832.6	(\pm 971.5)	6061.0	(\pm 1259.6)
Maximum shift in a note across song (Hz)	3553.8	(\pm 765.7)	3826.9	(\pm 1325.4)
Average note frequency range (Hz)	1610.7	(\pm 255.0)	1748.1	(\pm 334.8)
Frequency slope	-0.1	(\pm 1.5)	-0.1	(\pm 0.8)
Temporal position of amplitude peak	2.7	(\pm 0.9)	2.6	(\pm 0.8)
Note diversity across song	86.0	(\pm 12.1)	77.5	(\pm 13.8)
Number of notes in song	30.1	(\pm 11.9)	26.7	(\pm 8.7)

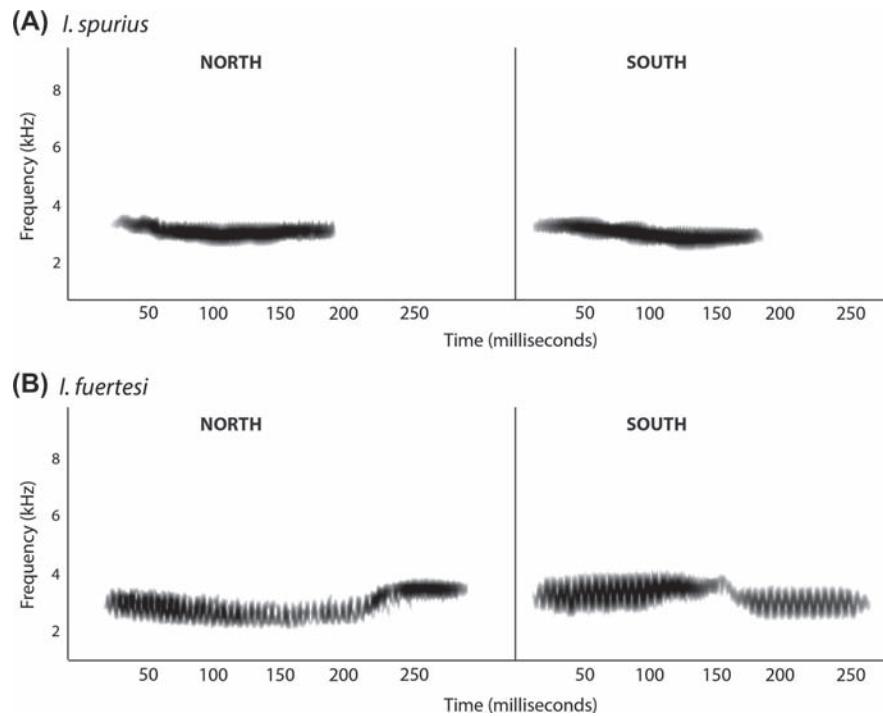


Figure 3. (A) Examples of *I. spurius* calls from the northern (Maryland, USA) and the southern (Texas, USA) populations included in this study. (B) Examples of *I. fuertesi* calls from the northern (north Veracruz, Mexico) and the southern (south Veracruz, Mexico) populations included in this study.

Statistical analyses

For the comparisons of songs between the two taxa, we conducted our statistical analyses using SPSS (released 2010, SPSS Statistics for Windows, ver. 19.0. Armonk, NY, IBM). All song characteristic variables were tested for homoscedasticity. Song duration and the frequency slope across the

Table 4. Untransformed means of jeet call characteristics measured for male *I. spurius* and *I. fuertesi*. Standard deviations are given in parentheses. Characteristics in bold are analogous to similar measures for the song (Table 3).

Call characteristic	<i>I. spurius</i>	<i>I. fuertesi</i>
Duration (ms)	173 (± 19)	241 (± 39)
no. parts in call	1.0 (± 0.0)	1.6 (± 38.6)
Maximum frequency (Hz)	3584 (± 145)	3734 (± 173)
Minimum frequency (Hz)	3022 (± 229)	2491 (± 200)
Frequency range across call (Hz)	562 (± 173)	1242 (± 215)
Overall peak frequency (Hz)	3178 (± 162)	3019 (± 304)
Beginning frequency (Hz)	3449 (± 131)	3336 (± 257)
Middle frequency (Hz)	3105 (± 198)	2942 (± 375)
End frequency (Hz)	3155 (± 182)	3177 (± 311)
Frequency modulation	20.1 (± 6.9)	113.8 (± 19.9)
Slope of frequency	-1.7 (± 0.7)	-0.9 (± 2.3)
Frequency ratios:		
Beginning/middle	1.1 (± 0.1)	1.1 (± 0.1)
Middle/end	1.0 (± 0.0)	0.9 (± 0.2)
Beginning/end	1.1 (± 0.0)	1.1 (± 0.2)
Amplitude ratios:		
Beginning/middle	1.4 (± 0.8)	1.6 (± 0.3)
Middle/end	2.7 (± 1.3)	2.9 (± 2.2)
Beginning/end	3.8 (± 2.6)	4.5 (± 3.9)
Amplitude modulation	3.3 (± 3.4)	9.0 (± 4.4)

song both failed equal variances tests, and so were analyzed separately from the other song characteristics. To control for correlations among the song variables that passed the equal variances tests, we used principal component analyses (PCA) to reduce the dataset into a set of orthogonal components. Principal components whose eigenvalues were at least 1.0 were then analyzed in a MANOVA to determine the amount of variation in songs that could be explained by species boundaries. Recordings were first grouped by taxon, and then further subdivided by region within each taxon to test for between population differences. Song recordings for *I. spurius* were grouped into western (Nebraska and Missouri), central (Illinois, Ohio, Ontario, Indiana), eastern (Maryland, New Jersey and South Carolina), and southern (Texas, Louisiana and Florida) regions. Recordings for *I. fuertesi* were grouped into northern (Mante, Tamaulipas and Tamiahua and Tuxpan, Veracruz) and southern (Las Barrancas and Tlacotalpan, Veracruz) regions. Breeding ranges of both species, as well as all recording locations for both songs and jeet calls are given in Fig. 1. Subsequent to the MANOVA, post hoc Fisher's LSD corrections were applied to pairwise comparisons of the principal components. The remaining two song characteristics (song duration and frequency slope) were analyzed using a Mann–Whitney U test, due to their lack of homoscedasticity.

To compare jeet call characteristics, we used an identical approach. We tested all variables for homoscedasticity. Overall call duration (ms) and frequency modulation (as measured by the standard deviation of frequency across the spectral contour) were both square-root transformed to ensure equal variances, while mean middle frequency (Hz), mean end frequency (Hz), number of parts to the call,

maximum frequency slope across the call, frequency range across the call, beginning/middle frequency ratio, middle/end frequency ratio, and beginning/end frequency ratio could not be transformed to meet the assumptions of parametric tests and were analyzed separately. We used PCA to reduce the remaining variables, along with the two that were square-root transformed, into orthogonal principal components. We then analyzed the principal components using a MANOVA to look for statistically significant differences between the two taxa. Call recordings were also grouped first by taxa, and then by regions within each taxon. As there were fewer recordings available for jeet calls compared with song, *I. spurius* recordings were grouped into northern (New York, New Jersey, Maryland, Indiana and Tennessee) and southern (Texas) regions. *Icterus fuertesi* recordings were again grouped into northern (Tamaulipas, Veracruz) and southern (Las Barrancas and Tlalocapan, Veracruz) regions (Fig. 1B). We tested principal component scores with univariate tests to determine which components differed significantly between *I. spurius* and *I. fuertesi*, using post hoc Fisher's LSD corrections. The call variables that failed the equal variances tests (listed above) were analyzed using a Mann–Whitney U test.

Data available from the Dryad Digital Repository: <<http://dx.doi.org/10.5061/dryad.469vn>> (Sturge et al. 2015).

Results

Song comparison

The means and standard deviations for song characteristic measurements are shown in Table 3. The PCA for song characteristics resulted in five principal components whose eigenvalues were greater than 1.0, and in total explained 73% of the observed variation (Table 5). The first two components together explained 37.5% of this variation. Song duration (seconds), and the number of notes within the song had

the strongest positive loadings on PC1, while average note duration (seconds) and the song's note diversity had the strongest negative loadings. For PC2, highest frequency across the song (Hz), frequency range across the song (Hz), maximum frequency shift across a note within the song (Hz) and the average note frequency range shift (Hz) all had strong positive loadings, while none of the song characteristics had strong negative loadings for this component.

A MANOVA comparing the principal component scores for *I. spurius* and *I. fuertesi* found no significant differences between the two taxa (Wilks' $\lambda = 0.916$, $p = 0.725$). Locations within each taxon also showed no significant differences (Wilks' $\lambda = 0.947$, $p = 0.881$). Therefore, we did not perform any univariate analyses on these principal components. Song duration and frequency slope across the song were both analyzed using a Mann–Whitney U test, and this test also failed to find any significant differences between the two taxa (song duration $U = 153$, $p = 0.422$; frequency slope $U = 278$, $p = 0.921$).

Call comparison

The means and standard deviations for jeet call characteristics are shown in Table 4. The PCA for call characteristics resulted in four components whose eigenvalues were greater than 1.0. These components explained 89% of the observed variation. The factor loadings for the call characteristics on each component, along with the variance explained by each component are shown in Table 6. Call duration (ms), frequency modulation and amplitude modulation all showed strong positive loadings on PC1, while the minimum frequency across the call (Hz) showed a strong negative loading. Beginning frequency (Hz) loaded moderately in a negative way onto PC1. This component explained 30.8% of the observed variation. PC2, which explained 26.0% of the variation, was associated strongly with average frequency (Hz), middle/end amplitude ratio and beginning/end amplitude ratio, while no call characteristics had a strong negative loading.

Table 5. Principal components analysis of temporal and spatial characteristics of songs. Values in bold represent strong factor loadings ($> \pm 0.6$).

Song characteristic	Factor loadings				
	PC1	PC2	PC3	PC4	PC5
Song duration (s)	0.705	0.155	0.258	0.004	0.425
Percentage of notes in song (%)	-0.122	0.010	-0.411	0.841	0.019
Percentage of note overlap (%)	0.150	0.068	-0.078	0.816	-0.252
Length of longest note (ms)	-0.474	0.252	0.392	0.300	-0.041
Length of longest pause (s)	0.114	0.027	0.904	-0.070	0.066
Average note duration (s)	-0.820	-0.038	0.223	-0.036	0.055
Average pause duration (s)	-0.212	0.018	0.828	-0.317	-0.149
Pause rate (pause/s)	0.584	-0.014	-0.586	-0.227	0.148
Overall peak frequency (Hz)	0.296	0.244	0.047	-0.206	0.486
Maximum frequency across song (Hz)	0.055	0.891	-0.014	-0.030	0.234
Minimum frequency across song (Hz)	0.121	-0.018	-0.109	-0.648	-0.226
Frequency range across song (Hz)	0.026	0.874	0.011	0.117	0.280
Maximum shift in a note across song (Hz)	0.197	0.865	0.047	0.013	0.009
Average note frequency range (Hz)	-0.203	0.753	0.070	0.030	-0.381
Temporal position of amplitude peak	-0.037	0.052	-0.123	0.106	0.743
Note diversity across song	-0.694	-0.054	-0.080	0.223	0.142
Number of notes in song	0.917	0.029	-0.085	0.235	0.210
Percent of variance explained	19.63	17.84	13.78	13.03	8.70

Table 6. Principal components analysis of temporal and spatial characteristics of calls. Values in bold represent strong factor loadings ($> \pm 0.6$).

Call characteristic	Factor loadings			
	PC1	PC2	PC3	PC4
Duration (ms)	0.914	-0.084	-0.121	-0.081
Maximum frequency (Hz)	0.172	0.059	0.958	-0.035
Minimum frequency (Hz)	-0.783	0.468	0.061	-0.312
Overall peak frequency (Hz)	-0.339	0.821	0.165	-0.185
Beginning frequency (Hz)	-0.501	0.124	0.724	-0.323
Frequency modulation	0.817	-0.048	0.452	0.244
Beginning middle amplitude ratio	0.012	0.101	-0.124	0.948
Middle/end amplitude ratio	0.110	0.938	0.041	0.110
Beginning/end amplitude ratio	0.118	0.814	-0.042	0.532
Amplitude modulation	0.738	0.351	-0.017	-0.009
Percent of variance explained	30.81	25.96	17.12	15.06

A MANOVA comparing the principal component scores for the two taxa found significant differences between the two taxa (Wilks' $\lambda = 0.170$, $p < 0.001$), but not between populations within taxa (Wilks' $\lambda = 0.965$, $p = 0.951$). After post hoc Fisher's LSD corrections, univariate pairwise comparisons found significant differences between the two taxa for only the first principal component ($F_{1,22} = 25.6$, $p < 0.001$)

(Fig. 4). As this component was largely driven by call duration (ms), the minimum frequency across the call (Hz), the beginning frequency of the call (Hz), and measurements of both frequency and amplitude modulation, it suggests that these variables differ significantly between the two taxa and could be used to separate them based on recordings of their calls. As a further confirmation of this result we performed separate t-tests on the raw variables for these acoustic characteristics, with post hoc Bonferroni corrections and found significant differences for: call duration ($t = 6.0$, $DF = 23$, $p < 0.001$), minimum frequency ($t = 6.0$, $DF = 23$, $p < 0.001$), frequency modulation ($t = 16.0$, $DF = 23$, $p < 0.001$), and amplitude modulation ($t = 3.6$, $DF = 23$, $p = 0.017$). In addition, evaluation of our non-homoscedastic variables non-parametric tests found that the frequency range across the call ($U = 1.0$, $p < 0.001$) and the number of parts of the call ($U = 24$, $p < 0.001$) both differed significantly between the two taxa (Table 7). The other six acoustic characteristics evaluated by Mann–Whitney U tests did not differ significantly between taxa (lowest p -value = 0.23).

Discussion

Song comparison

Bird vocalizations have the potential to diverge rapidly as taxa speciate – through sexual selection, through stochastic processes, and due to natural selection through different environmental or physical influences on the taxa

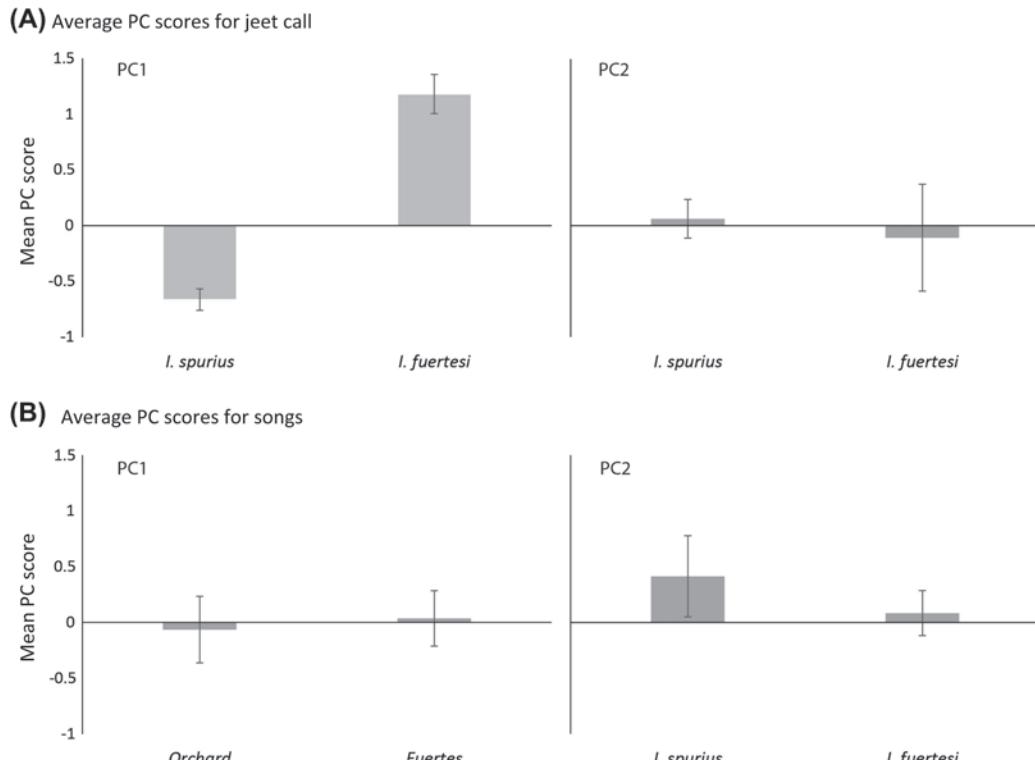


Figure 4. Average *I. spurius* and *I. fuertesi* scores (\pm SE) for the first two principal components of measurements taken for jeet calls (A) and songs (B). For jeet calls, call duration (ms), minimum frequency across call, and both frequency and amplitude modulations loaded most heavily onto PC1 (Table 6).

Table 7. Mann–Whitney U test of temporal and spatial characteristics of calls. p-values with * indicate significant differences ($p < 0.05$) after Bonferroni corrections are applied.

Call characteristic	U	p-value
Frequency range across call (Hz)	1.00	<0.001*
Middle frequency (Hz)	51.0	0.234
End frequency (Hz)	70.0	0.910
Number parts to call	24.0	<0.001*
Slope of frequency	54.0	0.308
Beginning/middle frequency ratio	61.0	0.533
Middle/end frequency ratio	54.0	0.308
Beginning/end frequency ratio	71.0	0.955

of interest (T. Price 1998, Kroodsma 2004, Marler 2004a, Catchpole and Slater 2008, Irwin et al. 2008, Derryberry et al. 2012). Recent studies have shown that closely related species, or even different subspecies, can show vocal divergence in songs or calls over a relatively short period of time (Seddon 2005, Dingle et al. 2010, Sewall 2011). New world oriole song comprises a labile suite of characters that can differ between closely related taxa (Price et al. 2007). Neutral genetic changes, on the other hand, may diverge much more slowly than traits under different selective pressures, creating the potential for very recently diverged taxa that are genetically indistinct at neutral markers to have fixed differences in their vocalizations (Isler et al. 1998, Irwin et al. 2001, Irwin 2009, Joseph and Omland 2009).

Icterus spurius and *I. fuertesi* are two such taxa. They lack reciprocal monophyly even in the fast sorting mtDNA, yet there is evidence of restricted gene flow between them (Baker et al. 2003, Sturge 2013). There is also some evidence that their nuclear DNA have begun to sort (Sturge 2013). However, in this study we found no evidence of statistically significant differences between the taxa in 18 acoustic song characteristics. In orioles, both song and plumage are highly pliable traits that have the capability of rapid divergence as both of these characteristics can evolve quickly through sexual selection as two taxa speciate (T. Price 1998, Price et al. 2007). Previous studies of *I. spurius* and *I. fuertesi* have found fixed differences in their adult male plumage coloration (Hofmann et al. 2007, Kiere et al. 2007), yet neither this study nor an earlier one examining syllable sharing between the taxa found evidence of song divergence (Hagemeyer et al. 2012). Thus, in this complex, plumage color appears to have diverged more rapidly than song, and is more likely to be acting as a potential reproductive barrier between the taxa (though it is possible that song has changed rapidly, and the resulting high variation causes sorting and divergence to take longer than for plumage color). It is also possible, however, that orioles are able to detect subtle differences between the songs of these two taxa – differences that our study may have failed to capture. In that case song might still act as a barrier to interbreeding.

Call comparison

Calls are vocalizations that have been described as acoustically simple, though in some groups they can be spectrally and temporally complex, and were traditionally thought to be entirely innate (Marler 2004a). While song in oscine passerines has long been known to involve a detailed learning process

(Catchpole and Slater 2008), a growing body of evidence has now shown that the acoustic characteristics of calls can be fairly plastic and subject to learning as well (Marler 2004b, Sewall 2009, 2011). Some call types, such as alarm calls, can be highly similar across species, due to the advantages of being able to elicit mixed species mobbing in response to predators for example (Ficken 2000). However, calls can also potentially diverge as two taxa speciate – either through different selective pressures or stochastic processes associated with the divergence event, or through cultural transmission of the parts of the call that are more plastic and subject to learning. Thus, examining calls between closely related taxa could also be informative and aid in our understanding of prezygotic barriers to gene flow.

The jeet calls included in this study show significant differences between *I. spurius* and *I. fuertesi* across a number of measured acoustic variables – suggesting that these calls may have diverged more rapidly than song. Our PCA of jeet calls resulted in four principal components with eigenvalues greater than 1.0, the first of which differed significantly between the taxa in the post-hoc pairwise univariate comparisons. Based on our results (Table 4), *I. fuertesi* produce longer calls that have lower minimum and beginning frequencies, and substantially more frequency and amplitude modulation than do the jeet calls of *I. spurius*. Frequency range across the call (Hz) differed significantly between these two taxa as well, with *I. fuertesi* having a much larger bandwidth than calls of *I. spurius*. By including different localities in this study (Fig. 1B), we can see that these differences do not match an isolation-by-distance alternative hypothesis: there were no significant differences between localities within each taxon. In other words, *I. spurius* jeet calls from Texas are more similar to jeet calls from Maryland than to the jeet calls of *I. fuertesi* in Veracruz, even though Veracruz is geographically closer to Texas than Maryland.

Our results also showed that the number of parts of this call also differed between the two taxa. *Icterus spurius* jeet calls were single notes, while many of the *I. fuertesi* calls appeared to be two-parted, including either a rise or a drop in frequency that was noticeable on spectrograms (Fig. 3). Both northern and southern populations of *I. fuertesi* included two-parted jeet calls, and more importantly, all *I. fuertesi* included in this study that produced one-part jeet calls also produced two-part jeet calls – suggesting that all *I. fuertesi* within our dataset were capable of producing jeet calls with two parts. None of the *I. spurius* in our dataset produced a two-parted call. Thus, the number of parts to the call provides a categorical distinction between the jeet calls of these two taxa.

Divergence in calls but not song

As the jeet call, but not song, appears to have diverged between our focal taxa, a call may be evolving more rapidly than song in this complex. Our results could be explained by either divergence in the genes controlling the expression of innate aspects of the jeet call, or cultural evolution of the potentially more plastic elements of these calls. Genetic changes associated with acoustic aspects of jeet calls could be correlated with stochastic processes occurring independently in the two taxa, or be related to ecological adaptations to their breeding ranges.

Martin and Omland (2011) found evidence of divergence in the bioclimatic niches of the breeding distributions of *I. spurius* and *I. fuertesi*, and the two taxa breed at different latitudes. Differences in either the abiotic or biotic aspects of their breeding habitats could conceivably result in different selection pressures leading to the observed variation in acoustic characteristics of their calls (Boncoraglio and Saino 2007, Ey and Fischer 2009). Reverberations are created when sound waves reflect from objects in the habitat, such as trees and leaves, while attenuation in an environment is related to both temperature and humidity – with the highest amount of attenuation occurring in areas with low humidity and high temperatures (Wiley and Richards 1978, 1982). Less modulated pure tones, such as the calls of *I. spurius*, are more robust to the effects of reverberations, particularly in highly vegetated (or ‘closed’) habitats (Wiley and Richards 1982, Dabelsteen et al. 1993, Slabbekoorn and Smith 2002), yet modulated acoustic signals are potentially more detectable than pure tones in a noise background (Klump and Langeman 1995, Lohr et al. 2003). Thus, the calls of *I. fuertesi* are likely more easily distorted by reverberations due to significantly greater amounts of frequency and amplitude modulation, but may have advantages in more open habitats (Dabelsteen et al. 1993). Variations in temperature and humidity between the breeding habitats of the two taxa, or the density and type of vegetation within those breeding habitats, could conceivably have led to differential evolution in the acoustic structure of the calls of these taxa since their divergence. Frequency and amplitude modulations in the jeet call are more rapid than those typically found in song (Fig. 2 and 3), and thus potentially more prone to selection due to the effects of environmental acoustics. However, it is possible that this ecological factor could potentially influence song structure as well as call structure.

Jeet calls may also be subject to sexual selection pressures due to their likely role in territory defense. Studies of red crossbill *Loxia curvirostra* ectomorphs have shown that calls are ecomorph-specific and have hypothesized that these calls could potentially aid in non-random mating, suggesting that calls have the potential to play a role in sexual selection (Sewall 2011). While *I. spurius* has a much larger winter range, the two taxa likely overwinter in sympatry in the southern portion of the range of *I. fuertesi*. If jeet calls, but not song, are used in the non-breeding season, it is possible that the divergence in call structure in particular could have resulted in part from character displacement resulting from sympatry during the winter. Such a pattern of divergence has been reported in the loudsongs of antbirds (Thamnophilidae), for example, which are more divergent in both temporal and frequency acoustic characteristics between closely related sympatric species than between ones that are allopatric (Seddon 2005).

Teasing apart the contributions of genetics versus culture, drift versus selection and natural versus sexual selection, on the structure of the jeet calls of these taxa lies beyond the scope of this study. However, our study raises many intriguing questions for future work. It is clear that the vocalizations of these two taxa show fixed differences, but unexpectedly in a call rather than in song. Song are acoustically distinct from calls in both taxa, with songs composed of many more individual elements. As a consequence, while we measured many

analogous acoustic characters in both songs and calls, it was impossible to measure an identical set of characters across both types of vocal signals. Similarly, our geographic comparison of songs and jeet calls were not identical given the more limited call dataset. It is possible that a broader sampling of song elements on a wider geographic scale resulted in greater variation that could have influenced our ability to detect consistent differences in song among *I. spurius* and *I. fuertesi*. However, our study, along with our previous work on song syllables (Hagemeyer et al. 2012), should have been able to detect any major song differences, if present. Our finding of significant differences in the characteristics of the jeet calls of *I. spurius* and *I. fuertesi*, over a range of sampling sites within each taxon is striking, and suggests that differences in the calls of these birds could potentially serve as prezygotic barriers to gene flow.

Future studies might address the plasticity in development of these respective vocalizations to answer questions about the role of learning in vocal variation in this group. Similarly, a wider phylogenetic approach could help elaborate on the lability of different vocalizations and vocal characteristics in orioles more broadly. Finally, a more exact analysis of habitat bioacoustics in these and other related taxa can answer questions about acoustic adaptation more directly, especially in this group, contributing to our overall understanding of the divergence between these two taxa in particular.

Conclusions

Icterus spurius and *I. fuertesi* have fixed differences in adult male plumage, in bioclimatic niches and in migratory behavior. They also have many similarities: they lack reciprocal monophyly in their mtDNA, and their female and juvenile plumage coloration and size morphology are similar. Based on their molecular genetics, these two taxa are in the earliest stages of speciation, making them an ideal focus group to examine species boundaries between sister taxa. Our study examined both male songs and jeet calls and found no evidence of divergence within the songs of these two taxa in the 18 acoustic measurements that we examined. However, we found statistically significant differences in call duration and number of parts to the call, in some of the frequencies across the call, and in the frequency and amplitude modulation. Therefore, while the songs of these two taxa failed to show evidence of differentiation, a call used in at least some similar contexts did show differences between the oriole taxa. Our study therefore highlights the possibility that calls may also be more evolutionarily labile, making them potentially useful vocal characteristics for examining pre-zygotic isolating mechanisms in early lineage divergence. Our work also reinforces the importance of studying calls to better understand their role in social contexts more generally (Marler 2004a). Vocalizations, including both song and calls, can be important metrics for understanding recently diverged species – both in terms of human diagnosability, and for understanding how recently diverged populations respond to one another (Mendelson and Shaw 2012).

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